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Issued September 6, 1910.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF PLANT INDUSTRY—Circular No. 67.
B. T. GALLOWAY, Chief of Bureau.

SEED STERILIZATION AND ITS EFFECT UPON SEED INOCULATION.

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54481°—Cir. 67—10

WASHINGTON : GOVERNMENT PRINTING OFFICE : 1910

BUREAU OF PLANT INDUSTRY.

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[Cir. 67]

B. P. I.—595.

SEED STERILIZATION AND ITS EFFECT UPON SEED INOCULATION.

INTRODUCTION.

As a preliminary to carrying on experiments in legume inoculation under control conditions, it is generally deemed advisable to disinfect the seed in order to prevent inoculation of the seedling roots through wind-borne or dust-borne organisms which might be present upon the seed. The subsequent effect of the sterilizing agents used does not seem to have been generally considered, despite indications that negative results secured in such tests have frequently been due to traces of the chemicals adhering to the seed coat.

STERILIZATION OF SEEDS.

To secure some definite knowledge of the effects produced by sterilization, some experiments were started in which a number of disinfectants were used and both leguminous and nonleguminous seeds were employed. Fifty seeds of each of seven different kinds were treated for varying periods in test tubes, rinsed several times with sterile water, and germinated in petri dishes. Equable moisture conditions were maintained by covering the seed with sterile filter paper of petri-dish size. Germination could then be noted without opening the petri dishes. To determine the germicidal effect of the treatments, platings were made on beef agar after sufficient time for germination had elapsed, a few seeds being shaken in with sterile water or the excess water in the petri dishes being used for plating. The germination and plating data are shown in the following tables:

TABLE I.—*Germination of seeds after treatment with various disinfectants.*

Seed.	Hydrogen peroxid, full strength.			Hydrogen peroxid, 1 per cent.[a]		Formaldehyde, 10 per cent.[b]		
	Check.	10 minutes.	60 minutes.	10 minutes.	60 minutes.	Check.	2 minutes.	15 minutes.
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Alfalfa	80	88	86	84	80	72	82	78
Crimson clover	88	86	62	66	68	70	76	0
Garden pea	74	96	76	94	96	94	50	60
Bean	86	90	90	90	80	90	26	0
Wheat	78	90	84	70	72	58	0	0
Corn	60	72	72	88	96	60	8	0
Radish	94	96	100	92	98	94	40	4

TABLE I.—*Germination of seeds after treatment with various disinfectants*—Continued.

Seed.	Formaldehyde, 1 per cent.		Mercuric chlorid, 0.5 per cent.			Mercuric chlorid, 0.1 per cent.	
	2 minutes.	15 minutes.	Check.	5 minutes.	30 minutes.	5 minutes.	30 minutes.
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Alfalfa	80	78	68	82	80	76	76
Crimson clover	84	82	76	62	4	76	74
Garden pea	84	80	96	96	84	98	96
Bean	88	10	90	94	78	90	90
Wheat	28	4	50	4	0	50	12
Corn	76	28	60	40	0	64	48
Radish	88	70	90	56	0	94	90

TABLE II.—*Effect of disinfectants in rendering seeds free from bacterial infection, the minus sign indicating the absence of infection.*

Seed.	Hydrogen peroxid, full strength.			Hydrogen peroxid, 1 per cent.[a]		Formaldehyde, 10 per cent.[b]		
	Check.	10 minutes.	60 minutes.	10 minutes.	60 minutes.	Check.	2 minutes.	15 minutes.
Alfalfa	+	−	−	−	−	+	−	−
Crimson clover	+	−	−	−	−	+	−	−
Garden pea	+	+	−	+	+	+	+	−
Bean	+	+	+	+	+	+	−	−
Wheat	+	−	−	−	−	+	−	−
Corn	+	−	−	−	−	+	−	−
Radish	+	−	−	+	+	+	−	−

Seed.	Formaldehyde, 1 per cent.		Mercuric chlorid, 0.5 per cent.			Mercuric chlorid, 0.1 per cent.	
	2 minutes.	15 minutes.	Check.	5 minutes.	30 minutes.	5 minutes.	30 minutes.
Alfalfa	−	−	+	−	−	+	−
Crimson clover	−	−	+	−	−	−	−
Garden pea	−	−	+	−	−	+	+
Bean	−	−	+	+	−	+	+
Wheat	−	−	+	−	−	−	−
Corn	+	−	+	−	−	+	−
Radish	−	−	+	−	−	−	−

[a] 1 per cent hydrogen peroxid =1 part commercial H_2O_2 (3 per cent) in 2 parts of water.
[b] 10 per cent formaldehyde =1 part of formalin (40 per cent) in 3 parts of water.

The figures concerning germination when taken alone do not indicate the actual differences shown by the various series. Many of the seeds treated with formaldehyde and mercuric chlorid produced distorted sprouts, showing various degrees of injury, while in the peroxid series the sprouts were usually larger and better in appearance than those in the untreated series, and the seed germinated more quickly.

So innocuous is the hydrogen peroxid that it may be used without appreciable injury on germinated seeds. For transplanting seedlings from one culture medium to another without transferring associated bacteria it has a special value as a sterilizing agent. It should be added that while hydrogen peroxid is an excellent bactericide it seems to have no more effect upon certain fungi, notably the Penicilliums, than it has upon the higher plants.

The leguminous seeds having hard coats withstood the toxic effect of formaldehyde and mercuric chlorid much better than the nonleguminous seeds, most of which were killed by the solution used. The failure to sterilize completely some seeds, e. g., pea and bean, by the same treatment with which other seeds were rendered sterile indicates either chance infections more resistant than those on the other seeds or the possibility that sometimes the infection exists under the seed coats. It has been authentically established in the case of bean anthracnose and loose smut of wheat that the seed itself is entered by the fungus. In many of our attempts to sterilize seed the conclusion seems inevitable that molds and fungi occur under the seed coats rather frequently in some lots of seed. Whether this is due to infection in the field or to improper storage has not been determined.

RESIDUAL EFFECTS OF SEED STERILIZATION.

To test the residual toxic action of disinfectants used on seed, some experiments were made by inoculating the wash water from treated seed. A preliminary experiment with pea, wheat, and radish seed treated with mercuric chlorid (0.5 per cent) for thirty minutes showed that the fourth wash water from the treated seed was decidedly toxic. After three times rinsing the seed in test tubes with sterile water, the next rinsing water was poured into a sterile tube and inoculated from a vigorous culture of *Bacillus subtilis*, tubes of sterile tap water being inoculated at the same time. Platings on beef agar were made two days later, and the plates after incubating for three days failed to show any growth from the wash water. The checks gave abundant colonies. It was evident that the mercury washed from the seed after three rinsings was still sufficient to be fatal to *B. subtilis* inoculated into the fourth wash water.

Similar tests were made in which wheat seed was treated with mercuric chlorid, formaldehyde, and hydrogen peroxid. The same methods were used, except that the peroxid-treated seeds were rinsed only once, the formaldehyde series twice, and the mercuric-chlorid series three times. Thus, the second, third, and fourth wash waters, respectively, were used in these tests. The results, showing the number of colonies of *Bacillus subtilis* surviving in the wash water, appear in Table III.

TABLE III.—*Growth of Bacillus subtilis in rinse water after disinfection of seed.*

Wheat seed treated with—	Time treated.	Strength of solution.	Number of times washed.	Bacillus subtilis present.
	Minutes.	*Per cent.*		*Colonies.*
Mercuric chlorid	30	0.5	4	0
Do	30	.1	4	200
Check				1,000
Formaldehyde	15	10.0	3	0
Do	15	1.0	3	50
Check				1,000
Hydrogen peroxid	60	3.0	2	0
Do	60	1.0	2	0
Check				1,000
Hydrogen peroxid	60	3.0	3	500
Check				1,000

It is evident that two rinsings were not sufficient to remove the hydrogen peroxid from the seed, and this part of the experiment was repeated, with one additional rinsing, giving the results indicated in the last two lines of the table. The third wash water showed slight inhibition in the growth of *Bacillus subtilis*, but nothing like as severe checking as with formaldehyde or mercuric chlorid.

The whole experiment was then repeated, flasks being used instead of test tubes. Bean seeds, fifty to each flask, were used. Each lot was rinsed three times and allowed to stand in sterile water fifteen minutes between rinsings. The flasks permitted the use of about five times as much water for rinsing as could be used in the test tubes, and the results showed practically complete removal of the disinfectants, at least in quantities toxic to *Bacillus subtilis*.

Brown[a] has shown that the seed coats of barley have the property of selective action in admitting or excluding various substances used for sterilization, e. g., sulphuric acid and copper sulphate are unable to diffuse into barley seed while mercuric chlorid penetrates readily. Acting on this suggestion, barley seeds (fifty in each lot, uniform in size and appearance and showing absence of external injury) were treated in test tubes for one hour and washed with sterile distilled water. The fourth wash water was then inoculated with *Bacillus subtilis*. Platings made two days later on beef agar gave the following results:

TABLE IV.—*Residual effect of disinfectants on barley seed.*

Treatment.	Strength of disinfectant.	Bacteria present.
	Per cent.	*Number.*
Sterile water		10,000
Sulphuric acid	25.0	50
Copper sulphate	10.0	250
Mercuric chlorid	.5	0
Formaldehyde	1.0	0

[a] Proceedings of the Royal Society (London), vol. 81, ser. B, no. B-546, 1909, p. 82.

From Table IV it appears that the sterilizing agents which are least able to pass through the seed coat into the seed were more nearly removed by the scant washing than those which could penetrate to the interior of the seed.

Hutchinson and Miller[a] have shown in an ingenious way that the persistence with which air bubbles remain on or inside the seed is responsible in many cases for incomplete sterilization. The use of a vacuum pump in connection with seed disinfection gave satisfactory, though not perfect, results.

As a means of sterilizing legume seed, this method seemed promising, but presented the possibility that there might be increased difficulty in freeing the seed from traces of the disinfectant. Accordingly, a test of the method was made, barley being used in addition to the seeds of several legumes in order to test further the semipermeable character claimed for the barley seed coat. It would naturally follow that poisons which penetrate the seed coats would be less completely removed by rinsing than those which are excluded by selective action.

Fifty seeds each of peas, beans, vetch, and barley selected for uniformity were treated by the vacuum method, and a like series by the usual method, shaking the seed up with the disinfectant in flasks. Each lot was rinsed four times, the last wash water (after standing fifteen minutes on the seed) being saved. This final wash water was then inoculated with *Bacillus subtilis* and allowed to stand two days, and platings were then made on beef agar. Counts made on these plates two days later indicated the toxic effect of the disinfectant left on the seed after rinsing. The seeds were germinated in sterile petri dishes by pouring into the dishes sterilized melted agar. An examination of the petri dishes one week later showed whether the seed had remained sterile or not.

No comparisons were made as to germination, except that a note was made when it appeared that the treatments had killed or injured the seed. Hydrogen peroxid (3 per cent), sulphuric acid (25 per cent), and mercuric chlorid (0.5 per cent) were selected for the disinfectants to be used, each lot of seed being treated for five minutes. In the vacuum series each solution was warmed to 40 to 50 degrees Centigrade, so that when a vacuum was applied the solution would boil. Air was admitted after a very brief boiling, and the solution allowed to act for five minutes. By connecting four bottles in series it was possible to draw off the disinfectant and to introduce the sterile wash water without opening the bottles. The results are given in Table V.

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[a] Journal of Agricultural Science, vol. 3, pt. 2, October, 1909, p. 179.

TABLE V.—*Residual effect of disinfectants on bean, pea, vetch, and barley seed.*

Treatment.	Seed infected or sterile.				Colonies of Bacillus subtilis developing after inoculation into wash water.			
	Pea.	Bean.	Vetch.	Barley.	Pea.	Bean.	Vetch.	Barley.
Vacuum series.								
Sterile water	++	+	++	+	500	1,000	600	200
Hydrogen peroxid	+	+	+	+	250	100	300	200
Sulphuric acid	+	+	−	−[a]	50	50	100	100
Mercuric chlorid	−[b]	−[b]	−	−[a]	0	20	75	0
Flask series.								
Sterile water	++	++	++	++	700	1,000	800	600
Hydrogen peroxid	+	+	+	−	350	800	400	125
Sulphuric acid	+	+	+	−[a]	300	700	250	50
Mercuric chlorid	+	+	−	−[a]	25	35	0	0

[a] Seed killed.

[b] Germination delayed, seed injured.

While there is no striking difference between the results obtained by the two methods, there is an evidently greater penetration of the disinfectant under the vacuum method of treatment. The beans treated with mercuric chlorid remained sterile with the application of a vacuum and at the same time were two days later in germinating. Throughout the two series there was less surviving contamination in the vacuum series than in the flask series.[a]

There was further evidence that the disinfectant was less completely removed with the vacuum treatment. In the peroxid treatments the average reduction of the number of *Bacillus subtilis* colonies was 63 per cent in the vacuum treatment and 46 per cent in the flask treatment; in the sulphuric-acid treatments the average reduction was 87 per cent for the vacuum series and 58 per cent for the flask series. Where mercuric chlorid was used the killing out of the inoculated organisms was so nearly complete that scarcely any difference exists between the two series.

The failure of barley to germinate after treatment with sulphuric acid was surprising in view of the results obtained by Brown (see p. 6), but this suggests that the impermeable character of the barley-seed covering may be abnormally developed in the case of the particular variety used by Brown. The effect on *Bacillus subtilis* of the wash water from barley again showed that sulphuric acid is more readily removed by rinsing than mercuric chlorid.

The experiments show that the removal of air bubbles by a vacuum does not entirely solve the difficulty in seed sterilization and does complicate the problem as regards the complete removal of the disinfecting solution. This latter factor is of prime importance in carrying on legume-inoculation experiments. The nodule organism

[a] By selecting seeds after germination on agar it is comparatively easy to secure sterile sprouts for transplanting to other culture media.

certainly could not withstand such quantities of toxic substances in solution as have proved fatal in these experiments to the more resistant *Bacillus subtilis*.

ACTION OF HYDROGEN PEROXID.

Small lots of peas, beans, and alfalfa seed in flasks or test tubes were treated for thirty minutes with full-strength commercial hydrogen peroxid (3 per cent), rinsed three times with sterile distilled water, and then inoculated with tested specific cultures of *Pseudomonas radicicola*. A duplicate series was washed with sterile water several times before inoculation; platings made from such washed seeds showed them to be nearly sterile, no bacteria and only a few molds developing. One lot of each kind of seed was dried by rolling in sterilized soil, the soil then being dusted from the seed; one lot was dried by contact with sterile filter paper; and one lot was placed in a desiccator, all seed being kept in sterile petri dishes. Platings were made by shaking ten seeds thoroughly with 10 cubic centimeters of sterile water, and plates were then poured with the nitrogen-poor synthetic agar (cane sugar, 10 grams; potassium phosphate, 1 gram; magnesium sulphate, 0.2 gram; agar, 15 grams; and tap water, 1,000 cubic centimeters) ordinarily used for growing *Pseudomonas radicicola*. The results were as shown in Tables VI and VII:

TABLE VI.—*Bacteria alive on culture-treated (sterilized) seed.*

Time when platings were made.	Bean seed.			Pea seed.			Alfalfa seed.		
	Dried in—			Dried in—			Dried in—		
	Soil.	Filter paper.	Desiccator.	Soil.	Filter paper.	Desiccator.	Soil.	Filter paper.	Desiccator.
	No.	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
Immediately after treatment	60	500	360	0	325	350	30	10	1,800
1 day after treatment	50	10	100	0	0	150	0	0	700
2 days after treatment	5	20	300	0	0	275	0	0	1,200
4 days after treatment	0	0	90	0	0	15	0	0	75

TABLE VII.—*Bacteria alive on culture-treated seed, previously washed with sterile water.*

Time when platings were made.	Bean seed.				Pea seed.				Alfalfa seed.			
	Dried in—				Dried in—				Dried in—			
	Soil.	Filter paper.	Open petri dish.	Desiccator.	Soil.	Filter paper.	Open petri dish.	Desiccator.	Soil.	Filter paper.	Open petri dish.	Desiccator.
	No.	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
Immediately after treatment	550	1,500	1,800	2,000	2	60	200	1,100	5	0	660	450
1 day after treatment	450	450	1,350	1,800	5	0	275	300	3	0	400	500
2 days after treatment	450	325	750	900	2	0	300	180	0	0	550	400
5 days after treatment	500	225	1,000	Mold.	0	0	180	Mold.	0	0	100	200

The rapid mortality of bacteria on the sterilized seeds shown in this series indicates that some influence was acting to weaken their normal vitality and that this varied with the vigor of the culture.[a] It further appears that the absorption of the liquid culture by the soil and by the filter paper used for drying removed the bacteria largely from the seed coats, especially in the case of peas and alfalfa. Seed dried in open petri dishes gave nearly as good returns as seed dried in the desiccator. These tests were repeated with practically the same results. A greater viability of legume bacteria on washed but unsterilized seed is reported in some similar tests by Edwards and Barlow.[b]

To test the possible removal of external contaminations by washing without disinfectants, several lots of leguminous seed (fourteen in all, of different kinds) were allowed to soak in sterile water for two hours, then rinsed twice, and the third water allowed to stand on the seed for one hour. Platings were made from the first and third waters, inoculating one loop into agar. Six of the fourteen lots gave sterile plates from the first water, doubtless due to the very small quantity of material used for inoculation. From the third water sterile plates resulted in every case but one, which had a single mold colony.

The seeds were allowed to soak two days in the third wash water to start any remaining organisms into growth. They were then placed in sterile petri dishes and germinated by pouring over them sterile melted agar. As was expected, nearly all of these tests gave plates somewhat contaminated—much less, however, than seed germinated similarly but without previous rinsing.

A third series of tests was then made, in which the vacuum method of washing was used, each lot of seed being rinsed five times. These seeds when germinated in agar showed less contamination than those in any of the preceding series, but a few organisms persisted in nearly every case.

As some of the plates showed colonies somewhat resembling the legume organism (*Pseudomonas radicicola*), a further test was made in which seed previously treated with pure cultures was used to make certain that *Pseudomonas radicicola* was originally present. One half of the treated seed was washed by the vacuum method, receiving, however, five rinsings instead of four. The other half was simply moistened and germinated, both lots of seed being germinated by pouring over them in sterile petri dishes melted nitrogen-

[a] Similar results showing increased resistance to killing out when legume bacteria were dried in a desiccator were reported by Kellerman and Beckwith (Science, n. s., vol. 23, 1906, pp. 471–472).

[b] Bulletin 169, Ontario Agricultural College, 1909, p. 15.

poor agar (see page 9), this medium giving the characteristic growth of *Pseudomonas radicicola.* A third lot of untreated seed was washed similarly to the treated seed and germinated under the same conditions.

The seed of six legumes (alfalfa, red clover, crimson clover, hairy vetch, wax bean, and Canadian field pea)—one hundred seeds in each of the three lots—was included in this test. Examination after four days showed *Pseudomonas radicicola* present on the culture-treated seed, both washed and unwashed. Comparisons showed that washing had been effective in removing about 75 per cent of the organisms placed on the seed, but those remaining were capable of vigorous growth. The untreated seeds, which were washed by the vacuum process, were rendered no more free from contamination than those rinsed by shaking in flasks or test tubes in the previous experiments. The number of colonies was much less than in either of the treated-seed series, but among these few (mostly of the *Bacillus subtilis* type) there again occurred occasional colonies closely resembling the typical form of *Pseudomonas radicicola.*

CONCLUSIONS.

As a preliminary to legume-inoculation experiments, seed disinfection should be practiced with special precaution.

Some method of treatment to eliminate chance contamination with the nodule organism is advisable in experiments where extremely accurate checks are necessary.

The usual methods of disinfection, employing metallic poisons such as mercuric chlorid, are objectionable, it having been shown that these poisons cling to seeds even after they have been rinsed in amounts sufficient to be harmful and often fatal to organisms placed on the seeds for inoculation.

Hydrogen peroxid has been found to be effective in eliminating bacterial contamination, and its residual effect is much less harmful than that of the other disinfectants tested.

Approved:

W. M. Hays,

Acting Secretary of Agriculture.

Washington, D. C., *July 22, 1910.*

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